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What stay-green mutants tell us about nitrogen remobilization in leaf senescence

Howard Thomas[1], Helen Ougham, Peter Canter and Iain Donnison

Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, Ceredigion SY23 3EB, UK

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Abstract

Leaf senescence has an important role in the plant's nitrogen economy. Chlorophyll catabolism is a visible symptom of protein mobilization. Genetic and environmental factors that interfere with yellowing tend to modify protein degradation as well. The chlorophyll–protein relationship is much closer for membrane proteins than it is for soluble or total leaf proteins. In stay-greens, genotypes with a specific defect in the chlorophyll catabolism pathway, soluble protein degradation during senescence may be close to normal, but light-harvesting and reaction centre thylakoid membrane proteins are much more stable. Genes for the chlorophyll catabolism pathway and its control are important in the regulation of protein mobilization. Genes for three steps in the pathway are reported to have been isolated. The gene responsible for the stay-green phenotype in grasses and legumes has not yet been cloned but a fair amount is known about it. Pigment metabolism in senescing leaves of the *Festuca–Lolium* stay-green mutant is clearly disturbed and is consistent with a blockage at the ring-opening (PaO) step in chlorophyll breakdown. PaO is *de novo* synthesized in senescence and thought to be the key enzyme in the chlorophyll *a* catabolic pathway. The stay-green mutation is likely to be located in the PaO gene, or a specific regulator of it. These genes may well be in the various senescence-enhanced cDNA collections that have been generated, but functional handles on them are currently lacking. When the stay-green locus from *Festuca pratensis* was introgressed into *Lolium temulentum*, a gene encoding *F. pratensis* UDPG-pyrophosphorylase was shown to have been transferred on the same chromosome segment. A strategy is described for cloning the stay-green gene, based on subtractive PCR-based analyses of intergeneric introgressions and map-based cloning.

Key words: Alien introgression, cDNA, chlorophyll catabolism, *Festuca*, *Lolium*, species-specific polymorphism, UDP-glucose pyrophosphorylase.

Introduction

Leaf senescence has an important role in the plant's nitrogen economy (Feller and Fischer, 1994). When demand from sinks is not met by current assimilation, stored N is remobilized. Once low molecular weight reserves have been drained, polymers begin to be catabolized. Leaves, and particularly chloroplasts, are sites of protein accumulation. Mobilization of chloroplast protein is a central metabolic activity in leaf senescence. Ordinarily, when leaves senesce they turn yellow. Chlorophyll catabolism is a visible symptom of protein mobilization in leaf senescence. In general, yellowing and protein N remobilization are well correlated. This is illustrated by Fig. 1, in which the chlorophyll $a+b$ content of *Lolium temulentum* leaf tissue measured at various times during senescence is plotted against total protein and shows a strong linear relationship ($P<0.05$). Genetic and environmental factors that interfere with yellowing tend to modify protein degradation as well. For example, a mutant gene that disables chlorophyll degradation was introgressed from *Festuca pratensis* into *L. temulentum* (Thomas *et al*., 1999). In this genotype, the broadly linear correlation between pigment and protein is still observed but the slope of the pigment–protein regression is markedly reduced (Fig. 1).

Most of the mobilizable protein in a leaf is soluble. Chlorophyll, on the other hand, is strictly confined to plastid membranes. The chlorophyll–protein relationship

[1] To whom correspondence should be addressed. Fax: +44 (0)1970 823242. E-mail: sid.thomas@bbsrc.ac.uk

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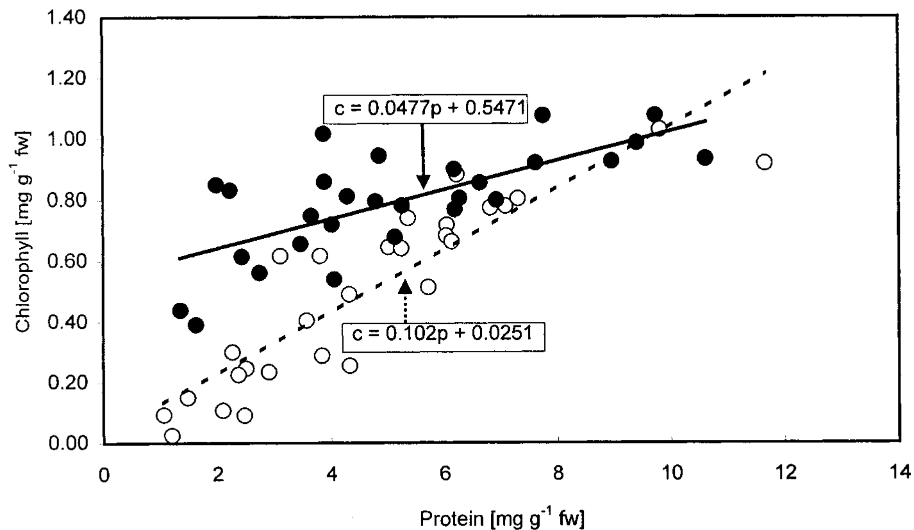

Fig. 1. Chlorophyll–protein relationships in senescing leaf tissue of wild-type *Lolium temulentum* Ceres (○) and of a stay-green introgression line (●). The data comprise observations from four independent experiments and fitted regression lines and equations are shown.

is much closer for membrane proteins than it is for soluble or total leaf proteins. If chlorophyll breakdown in senescence is prevented, soluble and membrane proteins diverge in their breakdown pattern. Figure 2 presents Western blots of a chlorophyll-binding thylakoid protein (LHCP2) and a typical soluble protein (GSAAT) from senescing leaf tissues of wild-type and stay-green introgression lines of *L. temulentum*. As described below, the latter genotype has a specific defect in the chlorophyll catabolism pathway. Degradation of soluble protein during senescence (exemplified by GSAAT) is close to normal. On the other hand membrane (light-harvesting and reaction centre) proteins are much more stable. Similar observations were made on stay-green lines of *Phaseolus vulgaris* (Bachmann *et al.*, 1994). *In vitro* experiments show that when pigment-binding proteins are not properly complexed with chlorophyll they are more vulnerable to proteolytic attack. One explanation for this is that the pigment facilitates protein folding so that the most resistant peptide regions are on the outside of the molecule. The behaviour of pigment metabolism mutants is consistent with a similar function for chlorophyll as a protein protectant *in vivo* (Thomas, 1997).

The pigment proteolipids of thylakoids have both a photosynthetic function and a role in membrane structure (Allen and Forsberg, 2001). Stabilizing chlorophyll–protein complexes in senescence has the effect of stabilizing membrane structure too. This in turn may reduce the lability of membrane-associated components that are not themselves directly stabilized by chlorophyll. An example of this is cytochrome *f* in chlorophyll catabolism mutants (Davies *et al.*, 1990*a*, *b*; Bachmann *et al.*, 1994). It also seems that a fraction of Rubisco that associates with the thylakoid can escape the fate of most

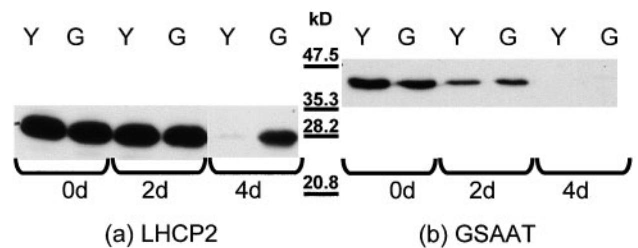

Fig. 2. Western blots of (a) light-harvesting chlorophyll protein of photosystem 2 (LHCP2) and (b) glutamic semialdehyde aminotransferase (GSAAT). Leaves of wild-type *L. temulentum* Ceres (Y) and an isoline homozygous for a stay-green introgression from *Festuca pratensis* (G) were incubated in darkness for up to 4 d and proteins were separated and detected according to Thomas *et al.* (Thomas *et al.*, 1999). The GSAAT antibody was the kind gift of Gamini Kanangara.

other stroma proteins in such plants (Davies *et al.*, 1990*b*). Conversely, part of a thylakoid intrinsic protein that extends into the stroma beyond the proteolytic no-go zone may be removed in non-yellowing mutants (Thomas and Howarth, 2000).

In the network of processes that regulate protein mobilization in senescence (Thomas and Donnison, 2000; Dangl *et al.*, 2000), the induction of chlorophyll degradation is an early and, for membrane polypeptides, essential event. Genes for the chlorophyll pathway and its control are therefore important in the regulation of protein mobilization. Chlorophyll catabolism is summarized in Fig. 3. The genes for three steps in the pathway have been cloned—chlorophyllase (Jakob-Wilk *et al.*, 1999; Tsuchiya *et al.*, 1999), RCC reductase (Wüthrich *et al.*, 2000) and the ABC-type tonoplast transporter (Lu *et al.*, 1998; Tommasini *et al.*, 1998). Mach *et al.* cloned the *ACD*2 locus of *Arabidopsis* and found it to be identical with RCC reductase (Mach *et al.*, 2001). The knockout has an accelerated cell death phenotype, taking

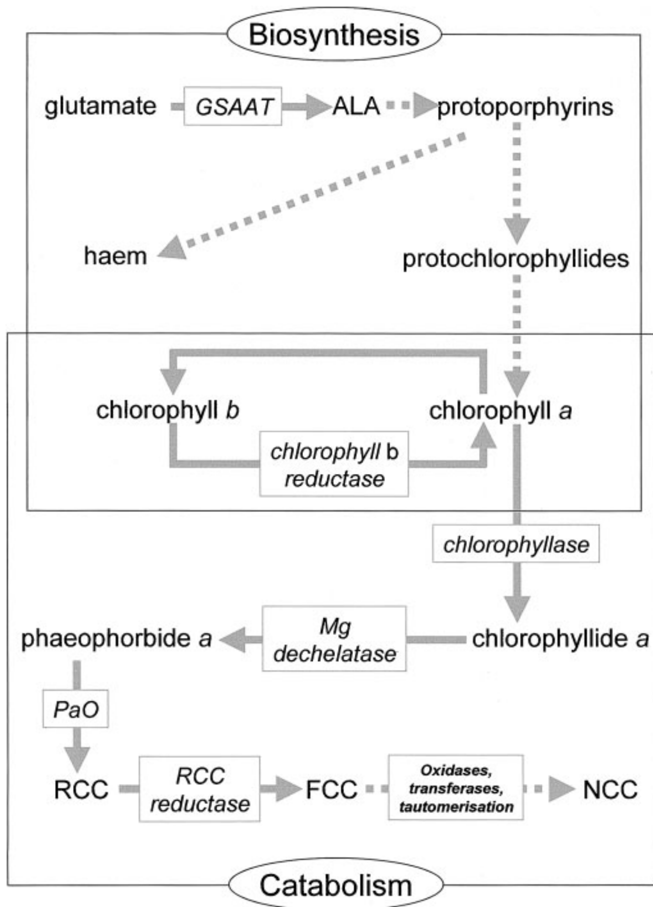

Fig. 3. A summary of chlorophyll biosynthesis and catabolism, indicating major intermediates and significant enzymes. GSAAT, glutamic semialdehyde aminotransferase; ALA, 2-aminolaevulinic acid; PaO, phaeophorbide *a* oxygenase; RCC, red chlorophyll catabolite; FCC, fluorescent chlorophyll catabolite; NCC, non-fluorescent chlorophyll catabolite. (Based on Thomas, 1997, and Matile *et al.*, 1999.)

the form of light-dependent spreading lesions, with no evidence of stay-green-type behaviour. The gene responsible for the non-yellowing phenotype in grasses and legumes has not yet been cloned but a fair amount is known about it. The rest of this paper will review the gene in *Festuca–Lolium* and will describe approaches to isolating it.

Stay-green *Festuca pratensis*

The non-yellowing trait in *Festuca pratensis* was shown to be inherited as a simple Mendelian recessive (Thomas, 1987). Superficially the phenotype seems to take the form of a more or less complete suppression of leaf senescence. Detailed comparison of leaves of the mutant with those of normally yellowing wild-type revealed the mutation to have interfered selectively with senescence processes. Total pigment levels decrease very little (but the complement of chlorophylls and their derivatives is altered in a characteristic way that is described later). Photosynthetic capacity diminishes more or less normally and the chlorophyll/$P_{\max}$ relationship of the mutant is very different from the wild type (Hauck *et al.*, 1997). The degradation of Rubisco and enhanced relative stability of chlorophyll-binding and other membrane-associated proteins are characteristic of senescence in the mutant (Thomas, 1987; Nock *et al.*, 1992). These features contribute to distinctive ultrastructural qualities (Thomas, 1977). Plastoglobuli in plastids of stay-green *F. pratensis* are less electron dense than in the wild type and their accumulation during senescence is much reduced. Thylakoids of the mutant persist in gerontoplasts as loosely-appressed, but still recognizable, membrane structures. Immunolocalization shows persistence of LHCP2 and its randomization throughout the surviving degenerate thylakoids (Hilditch *et al.*, 1989).

Special attributes of reproduction and genome organization in the two genera make species from the *Lolium–Festuca* complex particularly convenient subjects for introgression analysis. Introgression substitutes a gene by its homeologue from an alien background, allowing the locus to be identified, analysed and, ultimately, isolated. Species across the range of *Lolium* and *Festuca* are interfertile and homeologous chromosomes often pair and recombine freely (Canter *et al.*, 1999). Recombination between *Festuca* and *Lolium* species is generally promiscuous, with rates of gene substitution reaching more than 70% in some crosses. DNA introgressed by interspecific or intergeneric hybridization in *Lolium*/*Festuca* is detectable by genomic *in situ* hybridization (GISH; Thomas *et al.*, 1994) and by species-specific molecular markers (King *et al.*, 1998). Lines of *Lolium temulentum* have been produced with and without the stay-green introgression from *Festuca pratensis* (Thomas *et al.*, 1999).

Chlorophyll metabolism in stay-green *Festuca/Lolium*

Pigment metabolism in senescing leaves of *Festuca* and *Lolium* plants homozygous for the stay-green mutation is clearly disturbed. Chlorophyll *a* and total carotenoid levels are more or less stable during senescence, but chlorophyll *b* is almost as labile as it is in the wild type (Fig. 4). Dephytylated, more polar derivatives, largely chlorophyllide and phaeophorbide, accumulate in the mutant, but are virtually undetectable in the wild type (Thomas *et al.*, 1989). Assay of chlorophyllase, dechelatase, phaeophorbide *a* oxygenase (PaO), and red chlorophyll catabolite reductase (RCCR) show that PaO activity is missing in the mutant (Vicentini *et al.*, 1995). The pigment phenotype is consistent with a blockage at

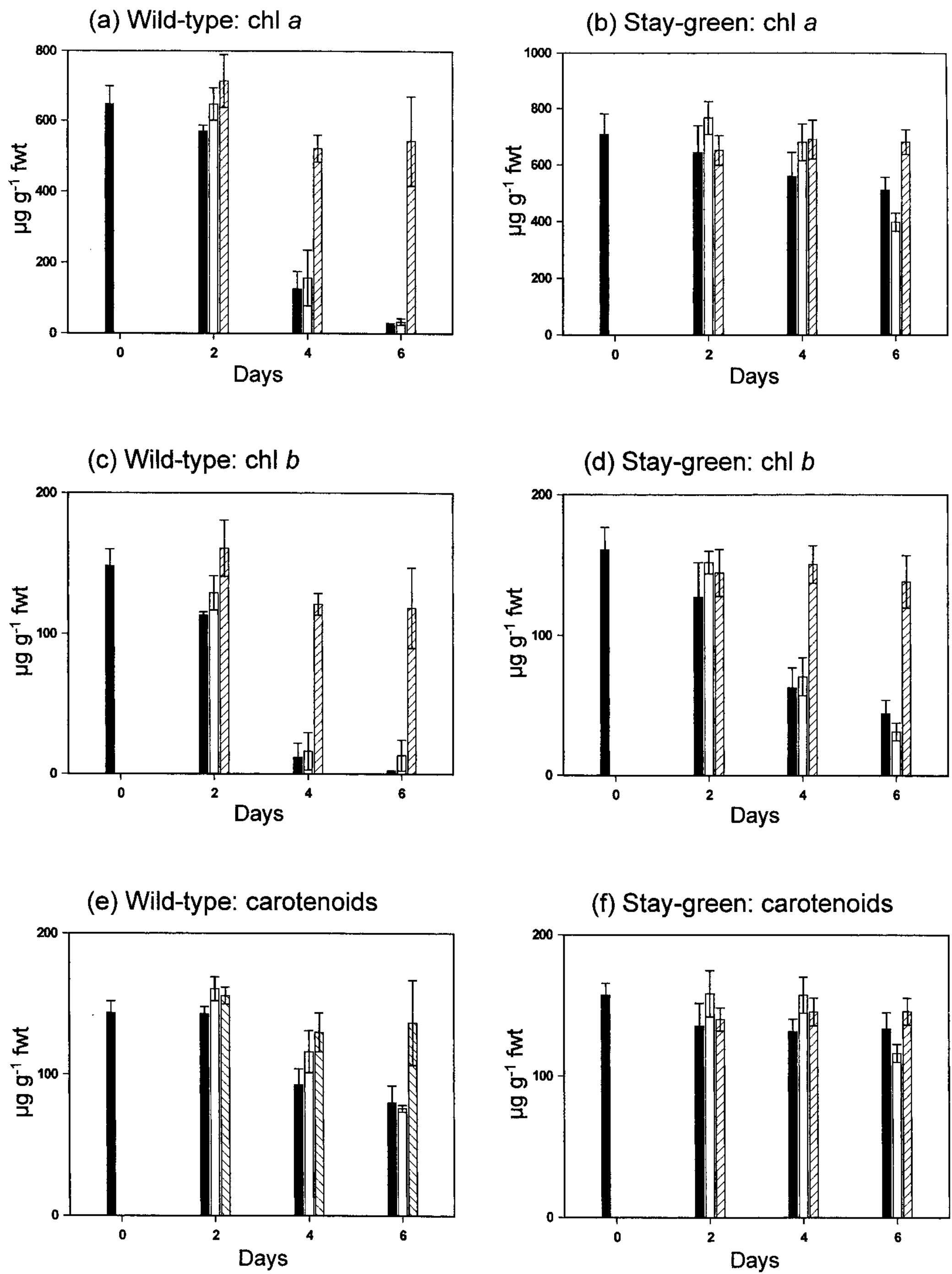

Fig. 4. Pigment contents of excised leaf segments senescing in the dark. (a, b) Chlorophyll *a*; (c, d) chlorophyll *b*; (e, f) carotenoids. (a, c, e) Wild-type *L. temulentum* Ceres; (b, d, f) homozygous for the stay-green introgression from *F. pratensis*. (■) Controls; (□) treated with the inactive L-stereoisomer of MDMP; (▨) treated with D-MDMP, an inhibitor of cytoplasmic protein synthesis. Error bars give standard deviations ($n = 4$).

PaO to RCC, resulting in progressive accumulation of proximal upstream catabolites. It should be mentioned that there is no evidence for disturbed chlorophyll biosynthesis in the mutant. As Fig. 2b demonstrates, chlorophyll biosynthetic enzymes, of which GSAAT is an example, are lost at normal rates during senescence.

Protein synthesis inhibitors prevent yellowing in senescing wild-type leaves. They also stop the pigment changes occurring in the stay-green mutant (Fig. 4). These data show that protein synthesis inhibitors act at two points in chlorophyll catabolism (Fig. 3). One is in the chlorophyll *b* to *a* conversion. Scheumann *et al.* showed that

chlorophyll *b* reductase activity increased several-fold during senescence of barley leaves (Scheumann *et al*., 1999). The response to translation inhibitor suggests that at least one of the two enzymes carrying out this conversion is synthesized *de novo*. The other point of inhibition is in the catabolic sequence from chlorophyll *a* to non-fluorescent chlorophyll catabolites (NCCs). No evidence was found for inhibitor-sensitive synthesis of chlorophyllase during senescence in *Lolium*. Chlorophyllase gene expression has been observed to be constitutive in a range of tissues, though ethylene and jasmonate had some modulating influence (Jakob-Wilk *et al*., 1999; Tsuchiya *et al*., 1999). Dechelatase (Langmeier *et al*., 1993) and RCCR (Wüthrich *et al*., 2000) are also constitutive. All the evidence points to PaO as the only enzyme in the chlorophyll *a* catabolic pathway to be *de novo* synthesized in senescence (Hörtensteiner *et al*., 1995) and the likely step at which translation inhibitors intervene (Rodoni *et al*., 1998). The phenotype of the stay-green *F. pratensis* and *Lolium* introgressions is consistent with a PaO knockout (Vicentini *et al*., 1995). The mutation may therefore be located in the PaO gene, or a specific regulator of it. Cloning the stay-green locus would give a gene with an important role in the internal N economy of green plants.

Genes expressed in stay-green *Festuca/Lolium*

Over recent years, differential and subtractive cloning has resulted in the isolation of large numbers of genes with senescence-enhanced expression patterns from a range of species (Buchanan-Wollaston, 1997; Dangl *et al*., 2000). Genes cloned from senescing leaves typically comprise a high proportion of sequences without a functional database match. For example, more than 2000 senescence-enhanced cDNAs (*See*s) have been isolated from maize. Of those sequenced, more than three-quarters are unique sequences and more than half have no significant database similarity. On the basis of such figures, it is quite likely that the wild-type gene corresponding to the *Festuca* stay-green locus is somewhere in one or more of the existing collections of senescence-related cDNAs. The problem is one of recognizing it in the absence of a clear functional handle. The unique features of introgression in *Lolium-Festuca* have been exploited to narrow the options and close in on the region of the genome that the stay-green locus occupies.

As well as promiscuous alien recombination, there is a further experimental benefit of *Lolium* and *Festuca* for genetic analysis: a high degree of molecular polymorphism between the species and genera. This is reflected not only in genomic sequences at given loci, but also in expressed genes. Expressed genes carried on an introgressed segment can be identified as species-specific polymorphisms in corresponding cDNAs. An example is UDP-glucose pyrophosphorylase (UDPGP). The stay-green gene from *F. pratensis* was transferred to *L. temulentum* Ceres and the alien segment reduced by several backcrosses (Thomas *et al*., 1999). Figure 5a shows that the small introgressed segment carrying the *F. pratensis* stay-green gene is located at the extreme end of one of the *L. temulentum* chromosomes. Genes expressed in wild-type and stay-green Ceres were compared by cDNA-RDA. Several differentially expressed genes were identified (Thomas *et al*., 1997) including one which, when sequenced, showed a good match to barley UDPGP (Fig. 5b, c). The same gene cloned independently from several stay-green and wild-type individuals showed distinctive phenotype-specific single nucleotide polymorphisms. In each of the sequences from *F. pratensis* (wild type or stay-green) and from stay-green *L. temulentum*, the nucleotides at positions 160, 450 and 458 are A, C and T, respectively. At the same positions in all sequences from wild-type *L. temulentum* G, T and G occur. Thus the UDPGP gene of *L. temulentum* plants carrying the single *F. pratensis* segment (Fig. 5a) is clearly a *Festuca* gene. It means that UDPGP is a gene linked closely to stay-green that has travelled all the way to *L. temulentum* Ceres on the same *F. pratensis*-derived alien segment. Other genes of similar origin will undoubtedly be identified within the collections of stay-green or wild-type-specific polymorphic fragments isolated by cDNA-AFLP (Table 1) and by cDNA-RDA (Thomas *et al*., 1997). For the purpose of seeking transcripts corresponding to the stay-green locus, introgression limits the numbers of candidates to those genes located on a small alien segment. Systematic functional testing, including complementation in transgenic *Lolium–Festuca*, thus becomes feasible. At the very least this approach generates several polymorphic markers close to the stay-green locus. Applied to a large-insert genomic library from *F. pratensis* (I Donnison and I King, unpublished results) together with genomic tags such as AFLPs and microsatellites, these open up the prospect of gene isolation by chromosome landing.

Conclusion

The stay-green gene from *Festuca pratensis*, and similar (homologous?) genes from other species including *Pisum sativum* (Thomas *et al*., 1996) and *Phaseolus vulgaris* (Ronning *et al*., 1991; Bachmann *et al*., 1994), profoundly influence the catabolism of both chlorophyll and protein during leaf senescence. Ever-closer cDNA and genomic tags for the *F. pratensis* stay-green locus have been identified. An *F. pratensis* BAC library is available. There is now detailed knowledge of the cellular and biochemical phenotype of stay-greens (Thomas and Howarth, 2000).

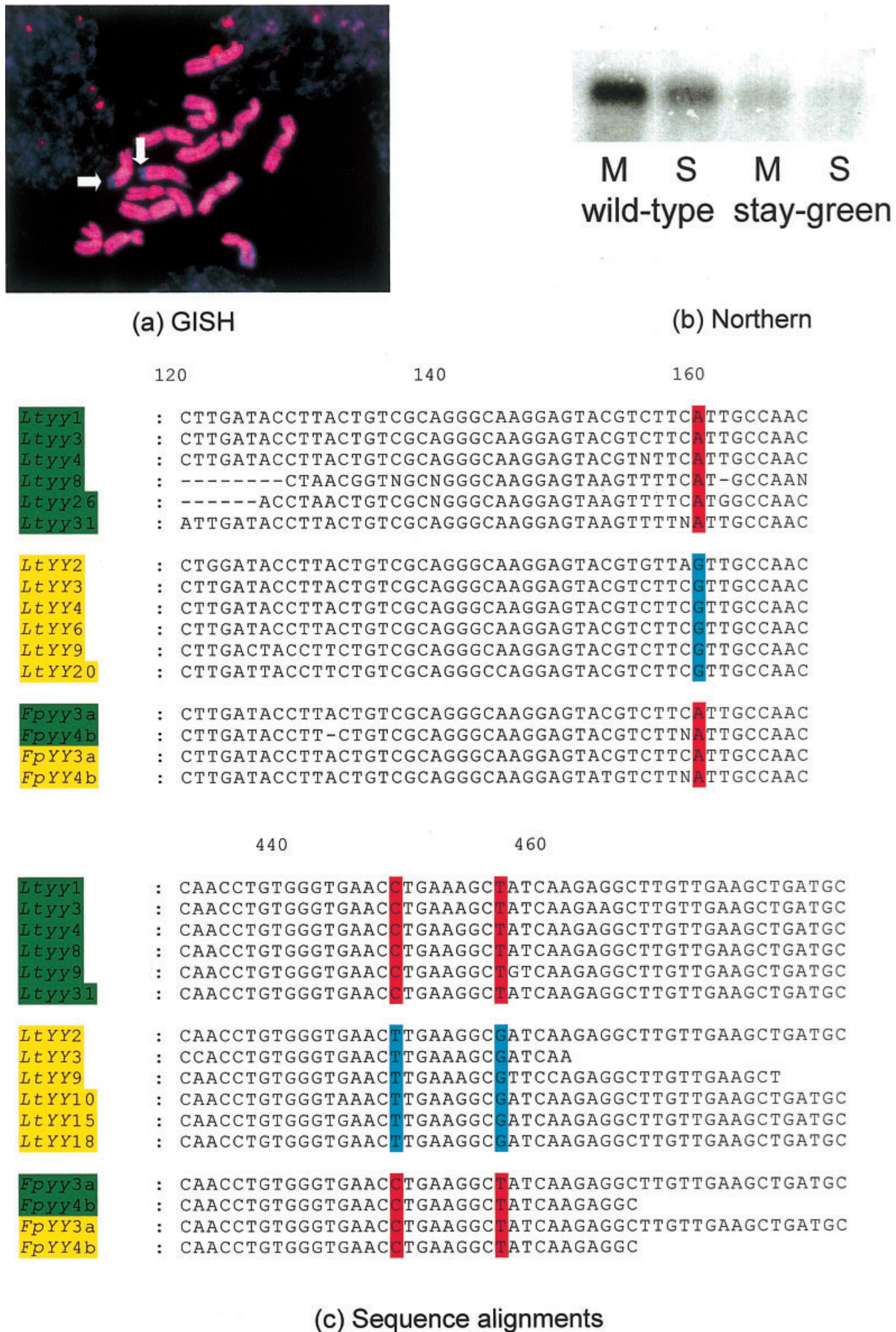

Fig. 5. (a) Genomic *in situ* hybridization of *Lolium temulentum* Ceres stay-green introgression line. The location of the pair of alien segments derived from *Festuca pratensis* is indicated. (b) Northern blot of RNA (10 µg per track) from mature (M) and senescing (S) *L. temulentum* leaf tissue hybridized with a cDNA for UDP-glucose pyrophosphorylase. (c) Alignments of UDPGP cDNA sequences independently isolated from stay-green (shown in green) and wild-type (yellow) *Lolium temulentum* (*Lt*) and *Festuca pratensis* (*Fp*). Single base polymorphisms of *F. pratensis* origin are shown in red, *L. temulentum*-specific in blue.

Table 1. *Clones, isolated by cDNA-AFLP, for genes showing differential expression between wild-type and stay-green* Lolium temulentum *leaves after 2 d senescence*

bp[a]	%[b]	Over[c]	WT/SG[d]	Homology[e]
450	58	135	WT	*Arabidopsis* putative transmembrane protein G1p
434	97	264	WT	*Zea mays* defender against cell death 1 (dad1) mRNA
319	64	110	WT	*Z. furfuracea* mRNA for vicilin (7S globulin)
403	61	155	WT	*Arabidopsis thaliana* MOM gene
400			WT	Unknown
365			WT	Unknown
299			WT	Unknown
359	65	211	SG	*Zea mays* calmodulin-binding protein
364	76	213	SG	*Zea mays* ubiquitin-conjugating enzyme
325			SG	Unknown
389			SG	Unknown
411			SG	Unknown
365			SG	Unknown

[a]bp: Length of clone in base pairs.
[b]%: Percentage DNA sequence homology.
[c]Over: length of region over which homology observed.
[d]WT/SG: more strongly expressed in wild type or stay-green.
[e]Homology: sequence in public databases to which greatest homology shown.

For functional testing, efficient homologous transformation systems are available in *Festuca* and *Lolium* species (Dalton *et al.*, 1999). Genomics resources in read-across models, notably maize, are directly accessible. It adds up to a set of powerful tools to isolate and characterize stay-green, which in turn will establish a crucial part of the regulatory mechanism for the internal N economy of plants.

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